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Incomplete replacement of docosahexaenoic acid by n-6 docosapentaenoic acid in the rat retina after an *n*-3 fatty acid deficient diet

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Abstract

When sources of *n*-3 fatty acids are not present in the diet, nervous system docosahexaenoic acid (22:6n3) is replaced by docosapentaenoic acid (22:5n6). Dams were fed either an *n*-3 deficient diet or one containing alpha-linolenic acid (18:3n3) and 22:6n3 throughout pregnancy and lactation. Their male offspring at weaning also received either the *n*-3 deficient or *n*-3 adequate diets and were sacrificed at 5, 10, 20, 50 and 91 days of age. Retinal lipids were extracted and analysed by gas chromatography for fatty acyl content. The percentage of retinal 22:6n3 increased continuously over the 13 week course of the experiment but reached its maximal concentration around day 20. Non-reciprocal replacement of 22:6n3 by 22:5n6 was observed at postnatal day 20 and 50 but not at other time points. Complete replacement of 22:6n3 was apparent if elevations in both 22:5n6 and docosatetraenoic acid (22:4n6) were considered. These data indicate that during the rapid period of accretion of retinal 22:6n3 around postnatal day 20, the supply of 22:5n6 to the retina was inadequate to completely replace 22:6n3 in *n*-3 deficient rats.
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Keywords: docosahexaenoic acid; docosapentaenoic acid; arachidonic acid; docosatetraenoic acid; *n*-3 fatty acid deficiency; retina; reciprocal replacement

1. Introduction

Docosahexaenoic acid (22:6n3) is the major polyunsaturated fatty acid of the nervous system including both the brain (O'Brien and Sampson, 1965; Crawford et al., 1976; Salem et al., 1986a,b; Salem, 1989) and retina (Anderson et al., 1974). The brain rapidly accretes 22:6n3 postnatally in humans (Clandinin et al., 1980; Martinez, 1992) and in rats (Green and Yavin, 1995; Moriguchi et al., 2004).

Generally, dietary deprivation of sources of *n*-3 fatty acids for two generations (Tinoco et al., 1978) has been used to induce a profound loss of nervous system 22:6n3. This loss of 22:6n3 is associated with an impairment of brain (Mohrhauer and Holman, 1963; Bourre et al., 1989; Yamamoto et al., 1991; Moriguchi et al., 2000; Greiner et al., 2001; Salem et al., 2001; Catalan et al., 2002) and retinal (Wheeler et al., 1975; Neuringer et al., 1986; Weisinger et al., 1996; Pawlosky et al., 1997) function. Recently, an artificial rearing model of *n*-3 deficiency has been developed that is capable of inducing a marked 22:6n3 deficiency in the first month of life in a rat and so such studies may now be performed on 'first generation' animals (Lim et al., 2003; Moriguchi et al., 2004).

In animal models of *n*-3 deficiency, diets are provided that contain linoleic acid (18:2n6) which is then metabolized such that *n*-6 docosapentaenoic acid (22:5n6) content increases in proportion to the 22:6n3 loss (Galli et al., 1971; Anderson et al., 1974; Tinoco et al., 1978; Neuringer et al., 1986; Salem et al., 1986a,b, 2001; Bourre et al., 1989; Salem, 1989; Yamamoto et al., 1991; Weisinger et al., 1996; Moriguchi et al., 2000, 2004; Greiner et al., 2001; Catalan et al., 2002; Greiner et al., 2003; Lim et al., 2003). This phenomenon was discovered several decades ago (Mohrhauer and Holman, 1963; Galli et al., 1971; Anderson

Abbreviations Arachidonic acid, (20:4n6); Dihomo-gamma-linolenic acid, (20:3n6); Docosatetraenoic acid, (22:4n6); Docosapentaenoic acid *n*-6, (22:5n6); Docosapentaenoic acid *n*-3, (22:5n3); Docosahexaenoic acid, (22:6n3); LC-PUFA, long chain polyunsaturates; Linoleic acid, (18:2n6); Alpha-linolenic acid, 18:3n3; *n*-3 Adq, *n*-3 fatty acid adequate; *n*-3 Def, *n*-3 fatty acid deficient; 22-C, 22 carbon; 20-C, 20 carbon.

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et al., 1974) and termed 'reciprocal replacement' by Galli et al. (1971). Since then, it has been widely assumed that there is in all cases of *n*-3 deficiency an efficient and complete replacement of 22:6n3 with 22:5n6.

However, the concept of reciprocal replacement of 22:6n3 has been based on an examination of the lipid composition of the brain or retina at adulthood. Recently, Greiner et al. (2003) observed that the replacement of 22:6n3 with 22:5n6 in the developing rat cortex was incomplete. That is, the sum of 22:6n3 and 22:5n6 in *n*-3 deficient rats did not equal that of *n*-3 adequate rats (fed both 18:3n3 and 22:6n3) on postnatal days 5, 10 and 20. However, complete reciprocal replacement of 22:6n3 with 22:5n6 did occur by postnatal days 50 and 91; another confirmation of the early work of Galli et al. (1971).

A crucial question for this field relates to the issue of what biochemical changes underlie the changes in retinal function associated with the *n*-3 deficiency syndrome. Can they simply be related to the loss of retinal 22:6n3? If so, then this entails a most extraordinary hypothesis that 22:5n6, a molecule only missing the double bond at the *n*-3 position with respect to the structure of 22:6n3, cannot functionally replace 22:6n3. A possible alternative is that 22:6n3 is not completely replaced during the rapid growth phase associated with early development. In this view, 22:5n6 may function as well as 22:6n3 but there is a lack of total 22-carbon polyunsaturates during rapid growth (Lands, 1992; Spector, 1999). Diminished levels of C22 polyunsaturates during neonatal development is associated with altered functions that may then lead to permanent changes in structure and impact some adult functions. It should be noted that in most studies of *n*-3 deficiency, the source of *n*-6 fatty acids in the diets is linoleic acid (18:2n6). Thus, 18:2n6 must be elongated/desaturated to 22:5n6 and transported to the nervous system membranes during active neurogenesis.

The present study examines the rat retina during *n*-3 fatty acid deficiency at various ages to determine to what extent 22:5n6 as well as other *n*-6 polyunsaturates replace 22:6n3 at various stages of postnatal development.

2. Methods

2.1. Animal treatment and study design

This study was approved by the Animal Care and Use Committee of the NIAAA, NIH. Two separate cohorts of Long Evans, female rats (Charles River, Portage, MI) were procured at 21 days of age. They were matched for weight and randomized into two dietary groups. Food and water were given in an ad libitum fashion. Animals were maintained under conventional conditions in our animal facility with controlled temperature (23 ± 1°C) and illumination (12 hr d^{-1}, 07:00–19:00 hr, 80 lumens). At about 11 weeks of age, the female rats were mated and the male offspring were used for this study. The pups were maintained on the same diet as their dams after weaning.

In order to maintain the greatest independence for each animal in a given group, each animal was selected from a different litter. Thus the *n* number signified the number of male rats from independent litters. Groups of rats were sacrificed at 0, 5, 10, 20, 50 and 91 days in order to measure lipid composition at various stages of development. Rat pups were sacrificed by decapitation and the retinas dissected out and frozen at −80°C.

2.2. Experimental diets

The two custom diets used in this study were based on the composition of the AIN-93 diet (Reeves et al., 1993) and prepared commercially (Dyets, Bethlehem, PA) as previously described (Greiner et al., 2003). However, several substitutions were made in order to meet the requirement of *n*-3 deficiency. For the casein source, vitamin-free casein (20/100 g diet) was used and corn starch was replaced with dextrose, cornstarch, maltose-dextrin and sucrose (20, 15, 15 and 10/100 g diet, respectively). Both diets contained (in g/100 g diet) cellulose (5), vitamin mix (1), salt-mineral mix (3.5), L-cystine (0.3), choline (0.25) and fat sources (10) as previously described (Greiner et al., 2003). The *n*-3 deficient diet (*n*-3 Def) contained 8.1 g hydrogenated coconut oil and 1.9 g safflower oil per 100 g diet. The *n*-3 adequate diet (*n*-3 Adq) contained 7.43 g hydrogenated coconut oil, 1.77 g safflower oil, 0.5 g flaxseed oil and 0.3 g of DHASCO™ (containing 46% of fatty acids as 22:6n3, Martek Biosciences Corp., Columbia, MD) per 100 g diet. This formulation provided a balanced intake of monounsaturates (3.5 and 4.6% in the *n*-3 Def and *n*-3 Adq diets, respectively) and 18:2n6 (15.5 and 15.2%) with the 3.0 wt% 18:3n3 and 1.5 wt% 22:6n3 in the *n*-3 Adq diet compensated for by saturated fat in the *n*-3 Def diet. The *n*-3 Def diet contained only 0.05% of fatty acids as 18:3n3; this originated from the safflower oil but also was a minor component in the protein source.

2.3. Fatty acid analysis

Retina samples were thawed and the lipids extracted according to the method of Folch et al. (1957) with 50 μg mL^{-1} butylated hydroxytoluene added as an antioxidant. An aliquot of each total lipid extract was then transmethylated using the method of Morrison and Smith (1964) as modified by Salem et al. (1996a,b). Methyl esters were quantified on a model 5890 series II gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector and a fused silica capillary column (DB-FFAP, 30 m × 0.25 mm × 0.25 μm, J&W, Folsom, CA). Hydrogen was used as carrier gas at a linear velocity of 50 cm s^{-1} and with injector, detector and initial column temperatures of 250, 250 and 130°C. The oven temperature program was 130–175°C at 4°C min^{-1},

175–210°C at 1°C m^{-1} and then to 245°C at 30°C m^{-1} with a final hold for 15 min. Tissue fatty acids were compared to the retention times of a quantitative standard (462, Nu-Chek Prep, Elysian, MN) and the concentrations of fatty acids were determined by reference to an internal standard (22:3n3 ethyl ester, 26–80 μg/sample depending upon age).

2.4. Statistical analysis

Data were expressed as the mean ± S.E.M. and analysed for statistical significance using a software package from Statistica (StatSoft, Tulsa, OK). Differences between the two dietary groups at each time point were analysed using the Student's t-test and were considered significant when $p \leq 0.05$.

3. Results

There were no differences in body, brain or liver weights between the *n*-3 Adq or *n*-3 Def groups at any time point with the exception that at day 91, the *n*-3 Adq group body weight was significantly ($p < 0.05$) greater (589 ± 42 g) compared to the *n*-3 Def (511 ± 34 g). There were no significant differences between dietary groups with respect to total fatty acid concentration. The total fatty acid concentration was greater during early development as it was in the range of ~17–20 μg of fatty acids per mg retinal wet weight at 5, 10 and 20 days postnatal age. Thereafter, the retinal fatty acid content fell to about 11 μg mg^{-1} wet weight by 50 days of age and 8–10 μg mg^{-1} by 91 days of age.

Initially, at birth, the pups in the *n*-3 Def group already exhibited a significant loss of retinal 22:6n3 and an increase in 22:5n6 with the 22:5n6/22:6n3 ratio in this group being over 27-fold that of the *n*-3 Adq group (Table 1). Other *n*-6 polyunsaturates appeared elevated, but non-significantly so in the *n*-3 Def pups. Although the sum of 22:6n3 + 22:5n6 had declined from 6.4 to 5.8 (*n*-3 Adq vs. *n*-3 Def), this difference was non-significant at birth. By postnatal day 5, the total lipid extracts of rat retinas in the *n*-3 Def group had a substantially lower percentage of 22:6n3 (1.2%) than did the *n*-3 Adq group (9.2%) with a 54-fold increase in the 22:5n6/22:6n3 ratio (Table 2). Accompanying this decrease in 22:6n3 in the *n*-3 Def group was a very substantial increase in 22:5n6 and significant increases in 22:4n6 and 20:4n6. The sum of 22:6n3 + 22:5n6 was somewhat lower in the *n*-3 Def group (9.52 ± 0.22) relative to the *n*-3 Adq (10.38 ± 0.42) although this difference did not reach statistical significance. When the sum of the 22:6n3 + 22:5n6 + 22:4n6 was considered, the mean values for the two dietary groups were then quite close. Similarly, when 22:5n3 was summed with these other 22-C polyunsaturates, there was no difference between dietary groups. It was interesting to note, however, that when 20:4n6 was summed with these other 22-C polyunsaturates, that the *n*-3 Def group had a significantly higher mean value. There were no differences in saturates, oleic acid or total monounsaturates between dietary groups.

Table 1
Rat retinal fatty acyl composition in the second generation of rats consuming an *n*-3 adequate or *n*-3 deficient diet at 0 days of age

Fatty acid	*n*-3 Def (*n* = 7)	*n*-3 Adq (*n* = 5)
Non-essential		
16:0	17.45 ± 1.54	19.51 ± 0.93
18:0	11.16 ± 1.08	12.91 ± 1.31
Total saturates	32.40 ± 2.80	36.63 ± 2.38
18:1n7	4.20 ± 0.33	4.52 ± 0.39
18:1n9	11.36 ± 0.90	12.08 ± 0.76
Total monounsaturates	20.61 ± 1.09	22.76 ± 1.29
n-6 polyunsaturates		
18:2n6	0.58 ± 0.18	0.80 ± 0.23
20:2n6	0.05 ± 0.02	0.04 ± 0.03
20:3n6	0.17 ± 0.03	0.29 ± 0.07
20:4n6	6.88 ± 0.90	6.34 ± 0.66
22:4n6	1.46 ± 0.26	1.20 ± 0.23
22:5n6	5.15 ± 0.44***	1.44 ± 0.16
Total *n*-6	14.44 ± 1.75	10.44 ± 1.34
n-3 polyunsaturates		
22:5n3	0.00 ± 0.00**	0.30 ± 0.05
22:6n3	0.62 ± 0.08***	4.95 ± 0.63
Total *n*-3	0.62 ± 0.02***	5.38 ± 0.55
(*n*-6) + (*n*-3)	15.06 ± 1.75	15.83 ± 1.36
22:5n6/22:6n3	8.90 ± 1.00***	0.32 ± 0.07
22:5n6 + 22:6n3	5.78 ± 0.47	6.40 ± 0.61
22:5n6 + 22:6n3 + 22:4n6	7.24 ± 0.68	7.60 ± 0.58
22:5n6 + 22:6n3 + 22:4n6 + 20:4n6	14.12 ± 3.80	13.95 ± 1.01
22:5n6 + 22:6n3 + 22:5n3 + 22:4n6	7.24 ± 0.68	7.91 ± 0.57
Total FA (μg mg^{-1} wet weight)	19.40 ± 1.37	22.79 ± 2.43

Data are expressed as the mean ± S.E.M. wt% of total fatty acids. Significance is indicated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. *n*-3 adequate group.

Much the same pattern of fatty acids was observed in the 10 day old rat retinas (Table 3). During the period from 5 to 10 days after birth, the mean percentage of 22:6n3 in the *n*-3 Adq group had increased from 9.2 to 17.7% with little change in the total fatty acid concentration. The percentages of two *n*-3 fatty acids, 22:6n3 and 22:5n3 were substantially decreased with compensation from the 20- and 22-C *n*-6 polyunsaturates in the *n*-3 Def group. Again the sum of 22:6n3 + 22:5n6 was lower in the *n*-3 Def group but was non-significant perhaps due to a limited number of samples at this time point. The sum of the 22:6n3 + 22:5n6 + 22:4n6 was again very similar between the two dietary groups. There was a slight increase in 16:0 and 18:1n7 in the *n*-3 Def group but no changes in the total monounsaturates between dietary groups.

Table 2
Rat retinal fatty acyl composition in the second generation of rats consuming an *n*-3 adequate or *n*-3 deficient diet at 5 days of age

Fatty acid	*n*-3 Def ($n=8$)	*n*-3 Adq ($n=7$)
Non-essential		
16:0	20.92±0.45	20.01±0.62
18:0	13.69±0.24	13.18±0.40
Total saturates	38.45±0.57	36.57±0.90
18:1n7	4.35±0.12	3.99±0.20
18:1n9	10.84±0.23	10.57±0.44
Total monounsaturates	19.40±0.35	19.43±0.64
n-6 polyunsaturates		
18:2n6	0.85±0.05	0.94±0.10
18:3n6	0.06±0.02	0.03±0.01
20:2n6	0.08±0.01	0.08±0.00
20:3n6	0.20±0.01***	0.25±0.01
20:4n6	10.35±0.20***	8.38±0.29
22:4n6	2.42±0.10***	1.37±0.07
22:5n6	8.31±0.22***	1.20±0.06
Total n-6		
n-3 polyunsaturates	22.30±0.34***	12.35±0.43
22:5n3	0.02±0.01***	0.47±0.02
22:6n3	1.21±0.07***	9.19±0.39
Total *n*-3	1.22±0.07***	9.70±0.40
(*n*-6)+(*n*-3)	23.52±0.37	22.05±0.74
22:5n6/22:6n3	7.05±0.44***	0.13±0.01
22:5n6+22:6n3	9.52±0.22	10.38±0.42
22:5n6+22:6n3+22:4n6	11.94±0.21	11.75±0.48
22:5n6+22:6n3+22:4n6+20:4n6	22.29±0.38*	20.14±0.74
22:5n6+22:6n3+22:5n3+22:4n6	11.95±0.22	12.22±0.50
Total FA (μg mg^{-1} wet weight)	17.03±0.86	19.70±1.15

Data are expressed as the mean±S.E.M. wt% of total fatty acids. Significance is indicated as follows: *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. *n*-3 adequate group.

Table 3
Rat retinal fatty acyl composition in the second generation of rats consuming an *n*-3 adequate or *n*-3 deficient diet at 10 days of age

Fatty acid	*n*-3 Def ($n=4$)	*n*-3 Adq ($n=4$)
Non-essential		
16:0	22.27±0.13*	21.33±0.27
18:0	16.60±0.04	16.21±0.25
Total saturates	43.53±0.18*	42.26±0.39
18:1n7	3.01±0.06*	2.81±0.03
18:1n9	8.99±0.17	8.98±0.14
Total monounsaturates	14.58±0.23	14.42±0.19
n-6 polyunsaturates		
18:2n6	0.83±0.05	1.17±0.20
18:3n6	0.00±0.00	0.00±0.00
20:2n6	0.11±0.01	0.12±0.00
20:3n6	0.25±0.01*	0.40±0.05
20:4n6	12.76±0.20***	11.03±0.29
22:4n6	2.54±0.04***	1.28±0.01
22:5n6	14.34±0.22***	0.68±0.05
Total *n*-6	30.83±0.29***	14.68±0.16
n-3 polyunsaturates		
22:5n3	0.06±0.00**	0.58±0.09
22:6n3	2.53±0.07***	17.74±0.88
Total *n*-3	2.59±0.07***	18.57±0.84
(*n*-6)+(*n*-3)	33.34±0.34	33.25±0.70
22:5n6/22:6n3	5.68±0.44***	0.04±0.00
22:5n6+22:6n3	16.87±0.32	18.41±0.88
22:5n6+22:6n3+22:4n6	19.41±0.35	19.69±0.88
22:5n6+22:6n3+22:4n6+20:4n6	32.17±0.31	30.72±0.95
22:5n6+22:6n3+22:5n3+22:4n6	19.47±0.36	20.28±0.84
Total FA (μg mg^{-1} wet weight)	17.05±0.55	16.75±0.50

Data are expressed as the mean±S.E.M. wt% of total fatty acids. Significance is indicated as follows: *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. *n*-3 adequate group.

The trend observed at 5 and 10 days postnatal age continued at 20 days of age (Table 4). By this time, the 22:6n3 had reached 27.6% of total fatty acids while the total fatty acid concentration was only slightly increased above the day 10 level. In the *n*-3 Def group, the 22:6n3 was only 4.3% but 22:5n6 was over 20% of total fatty acids and there were significant increases in 20:4n6 and 22:4n6 as well. However, the sum of 22:6n3+22:5n6 was significantly lower (by 12%) in the *n*-3 Def group relative to that of the *n*-3 Adq group. When 22:4n6 was added together with these fatty acids though, the mean values were not significantly different. There were no differences between saturates or monounsaturates between dietary groups except a slight increase in 18:1n7 in the *n*-3 Def group.

At 50 days postnatal age, a pattern much like that found in the 20 day retinas was evident (Table 5). The percentage of 22:6n3 in the *n*-3 Adq group had by then reached 31.5% of fatty acids although the retinal total fatty acid concentration had fallen considerably. In the *n*-3 Def group, 22:5n6 had reached almost 24% and both 20:4n6 and 22:4n6 were a significantly greater percentage of total fatty acids than in the *n*-3 Adq group. The sum of 22:6n3+22:5n6 was significantly lower in the *n*-3 Def group relative to that in the *n*-3 Adq group. When the sum of 22:6n3+22:5n6+22:4n6 was compared between the two dietary groups, no difference was observed. There were no significant changes in saturates or monounsaturates except a slight increase in 18:1n7 in the *n*-3 Def group.

At adulthood, at 91 days of age, the percentage of 22:6n3 in the *n*-3 Adq retinas had increased further to 34.3% and the total fatty acid content had decreased further (Table 6). The *n*-3 Def group still had a 22:6n3 level (8.6%) that was markedly lower than that of the *n*-3 Adq group. The level of

Table 4
Rat retinal fatty acyl composition in the second generation of rats consuming an *n*-3 adequate or *n*-3 deficient diet at 20 days of age

Fatty acid	*n*-3 Def ($n=5$)	*n*-3 Adq ($n=6$)
Non-essential		
16:0	17.78±0.57	17.85±0.29
18:0	18.92±0.48	18.76±0.41
Total saturates	38.86±0.99	38.70±0.65
18:1n7	1.88±0.07*	1.68±0.03
18:1n9	6.34±0.21	6.51±0.12
Total monounsaturates	10.28±0.39	10.19±0.59
n-6 polyunsaturates		
18:2n6	0.86±0.04**	1.15±0.05
18:3n6	0.00±0.00	0.00±0.00
20:2n6	0.18±0.01	0.20±0.01
20:3n6	0.25±0.02**	0.33±0.01
20:4n6	10.89±0.20***	8.42±0.25
22:4n6	3.39±0.10***	1.08±0.19
22:5n6	20.14±0.72***	0.35±0.01
Total *n*-6	35.75±0.98***	11.57±0.39
n-3 polyunsaturates		
22:5n3	0.06±0.02***	0.61±0.03
22:6n3	4.35±0.30***	27.64±0.66
Total *n*-3	4.41±0.29***	28.39±0.72
(*n*-6)+(*n*-3)	40.16±1.19	39.96±1.03
22:5n6/22:6n3	4.69±0.25***	0.01±0.00
22:5n6+22:6n3	24.49±0.94*	27.79±0.65
22:5n6+22:6n3+22:4n6	27.87±1.03	29.16±0.83
22:5n6+22:6n3+22:4n6+20:4n6	38.77±1.03	37.57±1.13
22:5n6+22:6n3+22:5n3+22:4n6	27.94±1.02	29.77±0.86
Total FA (μg mg^{-1} wet weight)	18.58±0.81	18.34±0.61

Data are expressed as the mean ± S.E.M. wt% of total fatty acids. Significance is indicated as follows: *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. *n*-3 adequate group.

Table 5
Rat retinal fatty acyl composition in the second generation of rats consuming an *n*-3 adequate or *n*-3 deficient diet at 50 days of age

Fatty acid	*n*-3 Def ($n=7$)	*n*-3 Adq ($n=8$)
Non-essential		
16:0	16.79±0.30	16.63±0.31
18:0	22.11±0.46	21.57±0.24
Total saturates	41.12±0.46	40.14±0.51
18:1n7	2.08±0.08*	1.89±0.04
18:1n9	6.57±0.14	6.82±0.13
Total monounsaturates	10.16±0.19	10.10±0.20
n-6 polyunsaturates		
18:2n6	0.57±0.03***	0.87±0.05
18:3n6	0.00±0.00	0.00±0.00
20:2n6	0.14±0.01	0.16±0.01
20:3n6	0.16±0.01***	0.26±0.01
20:4n6	9.12±0.16***	7.80±0.16
22:4n6	2.69±0.04***	1.17±0.03
22:5n6	23.91±0.78***	0.33±0.03
Total *n*-6	36.61±0.81***	10.57±0.20
n-3 polyunsaturates		
22:5n3	0.00±0.00***	0.49±0.01
22:6n3	5.34±0.24***	31.49±0.45
Total *n*-3	5.34±0.24***	31.98±0.45
(*n*-6)+(*n*-3)	41.95+/0.84	42.56/-0.38
22:5n6/22:6n3	4.53±0.23***	0.01±0.00
22:5n6+22:6n3	29.25±0.78*	31.82±0.46
22:5n6+22:6n3+22:4n6	31.94±0.79	32.83±0.51
22:5n6+22:6n3+22:4n6+20:4n6	41.07±0.85	40.69±0.46
22:5n6+22:6n3+22:5n3+22:4n6	31.94±0.79	29.12±4.70
Total FA (μg mg^{-1} wet weight)	10.59±0.87	11.32±0.98

Data are expressed as the mean ± S.E.M. wt% of total fatty acids. Significance is indicated as follows: *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. *n*-3 adequate group.

22:6n3 deficiency had diminished somewhat from that observed at 20–50 days of age as it had been an 83% decline but was a 75% decline by 91 days. In the *n*-3 Def group, the 22:5n6 had reached 26.2% of total fatty acids and 20:4n6 and 22:4n6 had also increased significantly with respect to the *n*-3 Adq reference group. At adulthood, there was no difference in the sum of the 22:6n3+22:5n6; thus, complete reciprocal replacement had finally occurred. Again, as observed at day 5, when 22:6n3, 22:5n6, 22:4n6 and 20:4n6 were summed, the *n*-3 Def group mean was significantly higher than that of the *n*-3 Def group.

4. Discussion

It has long been known that at adulthood, the 22:6n3 lost in the nervous system as a result of a prolonged dietary deficiency in sources of *n*-3 fatty acids is replaced by 22:5n6 (Mohrhauer and Holman, 1963; Galli et al., 1971; Anderson et al., 1974). This metabolic adaptation has been termed 'reciprocal replacement' of 22:6n3 with 22:5n6 (Galli et al., 1971). However, it has recently been shown that, in the cerebral cortex, the reciprocal replacement of 22:6n3 with 22:5n6 is not complete during the period of rapid brain growth between 5 and 20 postnatal days, as reflected by the sum of 22:6n3+22:5n6 (Greiner et al., 2003).

This non-reciprocal replacement of 22:6n3 by 22:5n6 is here, for the first time, demonstrated in the retina. However, the time course is distinct from that of the brain. The non-reciprocal replacement of 22:6n3 by 22:5n6 in the retina, although suggested by a lower mean level during the first 10 days of life, does not become statistically significant until postnatal day 20. It is quite possible though

Table 6
Rat retinal fatty acyl composition in the second generation of rats consuming an *n*-3 adequate or *n*-3 deficient diet at 91 days of age

Fatty acid	*n*-3 Def ($n=6$)	*n*-3 Adq ($n=6$)
Non-essential		
16:0	14.76±0.17*	15.57±0.31
18:0	21.28±0.20*	20.66±0.17
Total saturates	38.17±0.26	38.73±0.35
18:1n7	1.92±0.06	1.84±0.03
18:1n9	6.31±0.04	7.52±0.55
Total monounsaturates	10.24±0.22	11.08±0.61
n-6 polyunsaturates		
18:2n6	0.37±0.01***	0.56±0.04
18:3n6	0.00±0.00	0.01±0.01
20:2n6	0.09±0.01*	0.11±0.00
20:3n6	0.11±0.01***	0.23±0.02
20:4n6	8.73±0.08***	7.51±0.21
22:4n6	2.66±0.04***	1.22±0.05
22:5n6	26.17±0.53***	0.32±0.03
Total *n*-6	38.18±0.59***	10.03±0.26
n-3 polyunsaturates		
22:5n3	0.05±0.01***	0.43±0.03
22:6n3	8.60±0.13***	34.31±0.73
Total *n*-3	8.65±0.13***	34.85±0.74
(*n*-6)+(*n*-3)	46.83+/0.64	44.88±0.94
22:5n6/22:6n3	3.04±0.07***	0.01±0.001
22:5n6+22:6n3	34.77±0.57	34.63±0.75
22:5n6+22:6n3+22:4n6	37.43±0.60	35.66±0.78
22:5n6+22:6n3+22:4n6+20:4n6	46.16±0.64*	43.36±0.92
22:5n6+22:6n3+22:5n3+22:4n6	37.48±0.60	36.29±0.81
Total FA (μg mg^{-1} wet weight)	9.63±0.63	7.73±1.37

Data are expressed as the mean±S.E.M. wt% of total fatty acids. Significance is indicated as follows: *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. *n*-3 adequate group.

that with a greater number of subjects or for more detailed analyses of rod outer segment membranes or phospholipid molecular species, that the non-reciprocal replacement in the retina could be observed at the earlier time points. The sum of 22:6n3+22:5n6 remains significantly lower at 50 days of age albeit with a smaller difference. By the time that adulthood is attained by 91 days of age, there was no difference in the sum of 22:6n3+22:5n6.

Another manner in which to examine these data would be to examine the ratio made by summing 22:6n3 and 22:5n6 for the *n*-3 Def rat retinas and dividing by the same sum in the *n*-3 Adq rats. From day 5 to 50, this ratio is in the 0.88–0.92 range and only reaches 1.0 after 91 days. Although no longer time points were available in this study, these data are comparable to other data generated in this laboratory in past studies. For example, at 7 week of age, this ratio was 0.94 in the retinas of artificially reared rats, but after a subsequent 8 week period of repletion with *n*-3 fatty acids, the ratio was 0.99 (Moriguchi et al., 2001). In an artificial rearing study by Lim et al. (2003) at 8 week of age, the ratio was 0.95. In more aged animals, 33 week old, the ratio was then 0.98 (Weisinger et al., 2002). These data indicate that reciprocal replacement of 22:6n3 with 22:5n6 in the rat retina is a gradual process that extends into young adulthood.

The concept of reciprocal replacement of 22:6n3 with 22:5n6 should be extended to include other *n*-6 fatty acids. For the case of the retina, if both 22:5n6 and 22:4n6 are summed together with 22:6n3, then reciprocal replacement of 22:6n3 was complete at all time points over the course of this experiment. Thus, 22:4n6 must also be considered as an important fatty acid for reciprocal replacement of 22:6n3 in the retina. A similar situation occurred for the brain cortex for the 20 day time point as reciprocal replacement with 22:5n6 was incomplete (Greiner et al., 2003). However, in the brain, only when 20:4n6, 22:4n6, 22:5n6 and 22:6n3 were summed was there no difference between *n*-3 Adq and *n*-3 Def diet groups at any time point in the rat cortex. Thus, both 22:4n6 and 20:4n6 contribute to reciprocal replacement in the brain.

One might speculate that the differing time course for the non-reciprocal replacement of 22:6n3 in the retina and the brain may be related to the differing time courses for the normative accretion of 22:6n3 during retinal development. Initially, at birth, the retinal 22:6n3 percentage is low, unlike the high initial percentage observed in the brain (Moriguchi et al., 2004). In the retina, the percentage of 22:6n3 increased continuously with time over the 13 week course of this experiment. In terms of concentration, the peak in retinal 22:6n3 occurs at about 20 postnatal days when the concentration reaches 5.1 μg mg^{-1} wet weight. In contrast, this value is only about 1.8 μg mg^{-1} on postnatal day 5. The 20 day time point, where the accretion rate of 22:6n3 appears to be near maximal, coincides with the greatest degree of non-reciprocal replacement of 22:6n3 by 22:5n6.

An important question is what limits the supply of 22:5n6 to the retina such that it cannot replace 22:6n3 during this period with its maximal rate of accretion. One possibility is that the rate of 22:5n6 synthesis is inadequate. Since no *n*-6 precursors other than linoleic acid are supplied in the diet in this experiment, 22:5n6 must be biosynthesized from linoleic acid. Indeed, the in vivo biosynthesis of 22-C polyunsaturates occurs at a limited rate in humans (Demmelmair et al., 1995; Carnielli et al., 1996; Salem et al., 1996a,b; Hoffman et al., 2001). However, this possibility is tempered by the fact that the total complement of 22:6n3 found in the 20 day old rat retina is only about 91 μg. The serum and liver in the *n*-3 Def rat (at 7 weeks of age) contain a much larger pool of 1130 and 26 730 μg of 22:5n6, respectively (Moriguchi et al., 2001) and so it appears that a source of 22:5n6 is available. This then raises a

second possibility that the transport rate of 22:5n6 into the retina may be rate limiting. Indeed, transport of highly unsaturated fatty acids into the retina appears to be slow and rate limiting for the repletion of retinal 22:6n3 after a period of *n*-3 deficiency (Moriguchi et al., 2001). Similarly, the low rate of DHA transport into brain (Rapoport et al., 2001) is greatly diminished during *n*-3 fatty acid deficiency (Contreras et al., 2000).

Although this work demonstrates a failure to completely replace retinal 22:6n3 with 22:5n6 during early rat development, the limited magnitude of this effect may raise the question as to whether this effect is of functional significance. In vitro studies have conclusively demonstrated that rhodopsin function is dependent upon the number and positions of double bonds in membrane phospholipids (Litman and Mitchell, 1996; Litman et al., 2001; Mitchell et al., 2001; Mitchell et al., 2003a,b). It has been demonstrated that the substitution of 22:5n6 for 22:6n3 in phosphatidylcholine in liposomal membranes leads to a significant decrease in integrated signal transduction as measured by phosphodiesterase (Mitchell et al., 2001; Niu et al., 2001). Arachidonyl species of phospholipid are also less effective for rhodopsin activation than those of 22:6n3 (Litman and Mitchell, 1996). These studies have also recently been extended to the in vivo situation as rod outer segment membranes prepared from *n*-3 adequate or deficient rats exhibit similar changes in rhodopsin signaling (Niu et al., 2001). Furthermore, NMR studies have demonstrated that the biophysical properties of 22:5n6 are quite distinct from that of 22:6n3 (Eldho et al., 2003).

However, these in vitro studies generally compare the functions of membranes composed of a single phospholipid molecular species and it should be questioned whether a retinal membrane with only an approximately 12% loss of total 22:6n3 + 22:5n6 would be expected to exhibit altered function. It must first be recognized that retinal total lipid extracts were analysed here and so it is likely that a more detailed examination of lipid classes, cell types, and subcellular organelles such as the rod outer segment membrane where DHA is highly concentrated (Anderson et al., 1974) would reveal a more sizeable effect. Alterations in electroretinogram have been observed in rodents, cats, rhesus monkeys and human infants receiving vegetable oil-based formulas (Wheeler et al., 1975; Neuringer et al., 1986; Uauy et al., 1990; Birch et al., 1992; Weisinger et al., 1996; Pawlosky et al., 1997; Diau et al., 2003). Moreover, visual acuity (Neuringer et al., 1984; Makrides et al., 1995; Carlson et al., 1996a,b; Birch et al., 1998) and visual attention and visual recognition memory (Carlson and Werkman, 1996; Werkman and Carlson, 1996) are altered by feeding of infant formulas containing vegetable oil. These formulas contain some 18:3n3 but are devoid of 22:6n3 and thus a much milder state of 22:6n3 deficiency would be expected than the more radical rat model of *n*-3 deficiency where an attempt is made to remove all sources of *n*-3 fatty acids. Although it is unknown what retinal level of 22:6n3 and 22:5n6 was present in these infants, it is known that feeding a rat with a formula with a 10:1 ratio of 18:2n6:18:3n3 produced a 17% loss of retinal 22:6n3 with respect to well nourished, dam-reared rats (Woods et al., 1996). One autopsy study of infants fed vegetable oil-based diets observed losses of brain 22:6n3 relative to breast-fed infants but no significant change was observed in the retina (Makrides et al., 1994). These studies then suggest the possibility that moderate losses in retinal 22:6n3 may be associated with losses in retinal function. Apparently reciprocal replacement of 22:6n3 with 22:5n6 and 22:4n6 is not adequate for retinal function. It may then be inferred that the magnitude of losses of 22:6n3 + 22:5n6 observed in this study may impact retinal function.

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